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Cultural Performances of Two *Escherichia coli* Host-Vector Systems for Production of β-Galactosidase

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β-Galactosidase 생산을 위한 두 대장균 숙주-벡터의 배양 특성

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Protein productivities of a cloned gene (β-galactosidase) and the cultural performances of two recombinant *Escherichia coli* strains, which use different host-vector systems, were studied.

***E. coli* JM109/pTBG10 strain which carries *Tac* promoter had higher protein productivity than *E. coli* MH3000 (pRKcI857)/pASl(lacZ) strain which carries *pL* promoter. Induction of protein synthesis was optimum at the initial-and mid-logarithmic growth phases for both strains. Oxygen demand was observed to be very high during the cloned gene expression, and could be alleviated to some extent through pH control. The ratio of specific growth rates of plasmid-harboring to plasmid-free cell, $\mu+/\mu-$, of the high productivity strain was observed to be lower than that of the low productivity one. Plasmid stability was analyzed for 20-30 generations, and it was found that the fraction of plasmid-harboring cells dropped to 10% level in about 25 generations for both strains when the cloned gene expression was induced.**

Recent advances in genetic engineering and recombinant DNA technology made it possible to produce many "low volume, high value" proteins through the fermentation of recombinant microorganisms.

For the present, *Escherichia coli* strain is one of the most frequently used microorganisms, simply because its genetic information is greatly available. However, in many cases of foreign protein production using recombinant *E. coli* strains, genetic instability of the plasmid and produced protein (1) may cause a serious problem for the process development. Therefore, the selection of a proper host-vector system could be an important factor which may determine the economy of the fermentation process.

It has also been observed that the production of a fused protein, consisting of the desired protein and a polypeptide derived from β-galactosidase, allowed to overcome its genetic instability (2) and, furthermore, made the assay of the produced protein easy.

In our laboratory, during the course of process development for the production of hepatitis B vaccine, we have developed two recombinant *E. coli* strains with different host-vector systems, containing β-galactosidase gene as the model system.

The strain *E. coli* MH3000(pRKcI857)/pAS1 (lacZ), which uses *pL* promoter, was regulated by a temperature-sensitive cI repressor. Its gene expression was simply induced by temperature shift from 28 °C to 42 °C. The strain *E. coli* JM109/pTBG10, which uses *Tac* promoter, could be regulated by *lac* repressor, and the gene expression was induced by the addition of IPTG(isopropyl-thio-β-D-galactoside) at the concentration of 10^{-3}M.

Key words: β-Galactosidase, host-vector system, cultural performances
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In this study, regulation efficiencies of the gene expression and some cultural performances were compared by using these two recombinant *E. coli* strains.

Materials and Methods

Bacterial strains and plasmids

Escherichia coli MH3000[araD139, Δ(ara, leu) 7697, Δ(lac) 74, galU, galK, rpsL, (str^R), OmpR101] and JM109 [recA1, endA1, gyrA96, thi, hsdR, supE44, relA1, λ^-, Δ(lac-proAB), (F′ tra D36 proAB, $lacI^qZ$ M15)] were used throughout this study.

pRKcI857 plasmid which carries temperature sensitive repressor(cI ts) was introduced into the *E. coli* MH3000 strain, and MH3000(pRKcI857) was used as the host for pAS1(lacZ) plasmid. *E. coli* JM109 strain was used as a host strain for pTBG10 vector.

A general survey of the host-vector systems is shown in Fig. 1.

Medium and fermentation conditions

Luria broth(LB) which contains tryptone 10g, yeast extract 5g and NaCl 10g in 1*l* of distilled water was used as the growth medium. The pH was adjusted to 7.0 with 2N NaOH.

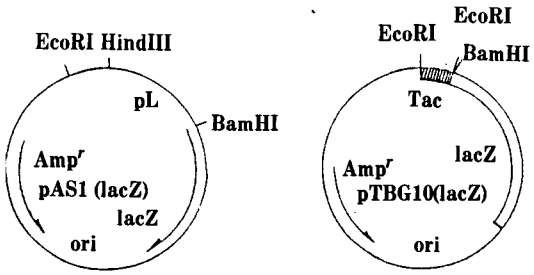

Fig. 1. Structure of pAS1(lacZ) vector and pTBG10 (lacZ) vector

- promoter: pL	- promoter: Tac
- gene: β-galactosidase	- gene: β- galactosidase
- regulation: CI^{ts} repressor/ heat induction (42°C)	- regulation: lac repressor/ IPTG induction (10^{-3}M)
- host: MH3000 (pRKCI857)	- host: JM109
- selection marker: Amp	- Selection marker: Amp
- replicon: pBR322	- replicon: pBR322

The medium was supplemented with 100μg/m*l* and 50μg/m*l* of ampicillin for seed culture and fermentation, respectively.

A single colony from LB agar plate supplemented with 200μg/m*l* of ampicillin was cultivated overnight at 28°C in 100 m*l* of seed culture medium, and this was then used as the inoculum for the fermentor operation.

Batch fermentations were conducted in a 2*l* Bioflo fermentor (New Brunswick Scientific) equipped with an oxygen analyzer and a pH controller.

E. coli MH3000(pRKcI857)/pAS1(lacZ) strain was initially cultivated at 28°C, and depression was made by shifting temperature to 42°C at the induction point. *E. coli* JM109/pTBG10 strain was cultivated at a constant temperature, 37°C, and derepression was made by the addition of IPTG at the concentration of 10^{-3}M.

Analytical methods

Cell concentration was measured by the optical density of the culture broth at 600nm. The apparent specific growth rate was estimated from the cell growth curve. Dissolved oxygen concentration was measured by using a New Brunswick sterilizable polarographic oxygen electrode and an oxygen analyzer DO-40(New Brunswick Scientific).

β-Galactosidase activity was measured by Miller method (3): after cell density was measured at 600 nm, aliquots of the culture broth were added to the assay buffer. (Z buffer: $Na_2HPO_47H_2O$, 0.06M; $NaH_2PO_4H_2O$, 0.04M; KCl, 0.01M; $MgSO_47H_2O$, 0.01M; β-mercaptoethanol, 0.05M; pH 7.0). The final volume should always be 1m*l*. Two drops of chloroform and 50μ*l* of 0.1% SDS solution were added to the assay mixture, and vortexed for 10 seconds. After placing the assay mixture in a water bath at 28°C for 5 minutes, the assay reaction was started by adding 0.2 m*l* of o-nitrophenyl-β-D-galactoside(ONPG) (4mg/m*l*) to the tube. The reaction was stopped by adding 0.5m*l* of 0.1M Na_2CO_3 solution after faint yellow color had developed. The reaction time was checked, and the optical density was measured at 420nm after centrifugation. Miller units of the sample was determined by the following formula

$$M.U. = 1000 \times \frac{OD_{420}}{t \times v \times OD_{600}}$$

where OD_{420} and OD_{600} represent the optical densities of the reaction mixture and the culture broth, respectively, measured at the specified wavelengths; t was the reaction time in minutes and v the volume in m*l* of the culture broth used in the assay.

In this assay, the color development, i.e., OD_{420}, was found to be linear under the specified assay conditions.

Stabilities of the recombinant microorganisms were determined based on the principle that plasmid-harboring cells produce β-galactosidase and form blue colonies on X-gal(5-bromo-4-chloro-3-Indolyl-B-D-Galactopyranoside) plates.

Precultured cells were diluted and inoculated into 100m*l* of LB medium in 500m*l* Erlenmeyer flask such that approximately 40 cells/m*l* could be obtained. Samplings were made at appropriate time intervals and they were spread on X-gal plate after dilution of 10^{-7} were made. The X-gal agar plate was incubated overnight at 28°. From the number of colonies appeared on the X-gal plate, plasmid stabilities were determined as the ratio of the number of blue colonies to the total number of colonies appeared.

Results and Discussion

Protein productivities of the recombinant strains

It is generally known that under the derepressed condition the fully induced promoter may lead to plasmid instability through interference with the replication mechanism. Efficient regulation of the cloned gene expression before the optimum induction point is thus important for more stable maintenance of the plasmid during the fermentation (4,5,6).

β-Galactosidase activity before and after induction was shown in Table 1. The strain B, *E. coli* JM109/pTBG10 showed higher productivity than the strain A, *E. coli* MH3000(pRKcI857)/pAS1 (lacZ), under the induced condition.

Table 1. β-Galactosidase activity before and after induction

strain	β-galactosidase activity (M.U.)*	
	before induction	after induction
Strain A; *E. coli* MH3000(pRKcI857) /pAS1(lacZ)	3,000	9,000
Strain B; *E. coli* JM109/pTBG10	2,000	62,000

* The data include maximum error of 12% and standard error of 8%.

Determination of the optimum induction point

Enhanced production of the foreign protein after induction inhibits growth of the cell (or lethal to the cell), and results in the plasmid instability during fermentation (4). Therefore it is important to optimize the induction point of gene expression for the optimum product yield.

To determine the optimum induction point for strain A, culture profiles of cell growth and protein production were first observed with the inductions at initial, mid, and late logarithmic growth phases (Fig. 2). It was noted that inductions at initial and mid-log phase yielded higher β-galactosidase activities (9,000 and 10,000 M.U., respectively) than that obtained from late-log induction (7,000 M.U.).

A similar productivity pattern was also observed for the strain B; 60,000 M.U. and 70,000 M.U. with initial and mid-log phase induction, respectively, while 40,000 M.U. with late-log phase induction (Data not shown). This could be explained by the fact that protein synthesis (or the gene expression) could effectively be carried out by actively growing cells (7). With the strain B, therefore, only the induction at initial growth phase was made (Fig. 3).

It was noted that oxygen demand of the cell was very high during the growth, resulting in the deficiency of dissolved oxygen concentration (Fig. 3(a)). Dissolved oxygen level in the culture broth was therefore considered to be important for the productivity of the system. In this regard, the effect of dissolved oxygen level on the protein production was thus investigated using the strain B. As shown in Fig. 3(b), when dissolved oxygen level was controlled at 40% of the saturated level by changing the agitation speed from 400 to 500 rpm, it was ob-

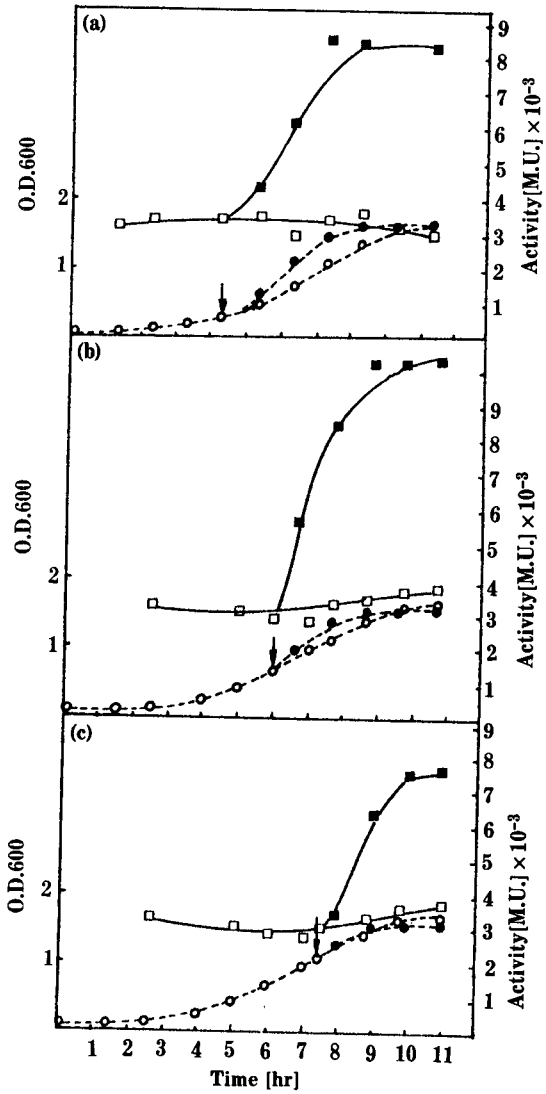

Fig. 2. Culture profiles of the strain A, *E. coli* MH3000 (pRKcI857)/pAS1(lacZ) with different induction times
Cell growth ○; 28°C
●; 28°C→42°C at↓
Activity □; 28°C
■; 28°C→ 42°C at↓
a) initial-log phase induction
b) mid-log phase induction
c) late-log phase induction

served that the protein production increased about 50%.

The decreasing tendency of β-galactosidase activity after its maximum was probably due to the loss of cell viability and the degradation of the produced protein.

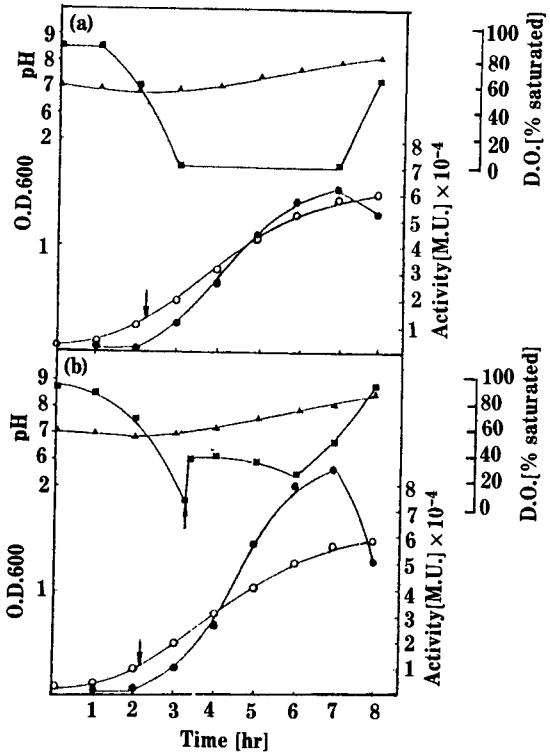

Fig. 3. Culture profiles of the strain B, *E. coli* JM109/pTBG10
○; Cell growth, ●; β-gal activity▲; pH ■; D.O.
a) initial-log phase induction at ↓
b) initial-log phase induction at ↓ : Agitation speed was increased from 500 rpm. at↑

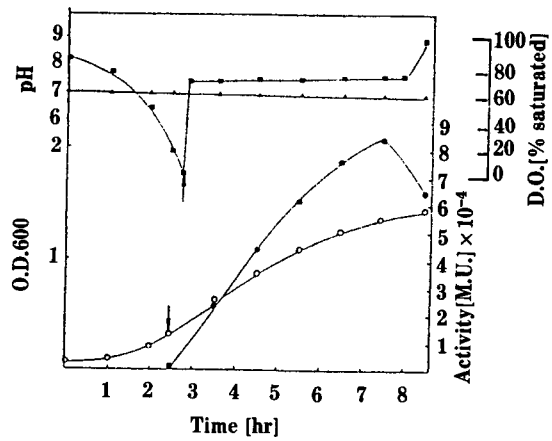

Fig. 4. Culture profiles of the strain B, *E. coli* JM109/pTBG10 with pH control at 7.0
initial-log phase induction at↓
Agitation: 400 rpm→500 rpm at↑
○; cell growth ●; β-gal activity▲; pH ■; D.O.

Table 2. Specific growth rates of MH3000 (pRKcI857)/pAS1(lacZ) and JM109/pTBG10(lacZ)

host cell	plasmid	Medium		Specific growth rates(hr^{-1}) plasmid(+) cells, μ^+	plasmid(-) cells, μ^-	μ^+/μ^-	Cultivation temp.
E. coli MH3000 (pRKcI857)	pAS1(lacZ)	LB	w/o ind.	0.43	0.47	0.92	28°C
			ind.	0.58	0.76	0.76	42°C
E. coli JM109	pTBG10(lacZ)	LB	w/o ind.	0.52	0.59	0.87	37°C
			ind.	0.37	0.59	0.62	

* w/o ind.: without induction
ind. : induced condition

Another experiment was conducted under the same protocol as in the Fig. 3(b), but at a fixed pH of 7.0 (Fig. 4). Under the control of pH it was noticed that oxygen demand was alleviated to some extent, which agrees with the work reported by Botterman *et al.* (7) that oxygen demand was lower at constant pH than that without pH control. At the present point, however, it is not clear how the cell metabolism and gene expression machinary can be affected under the condition of constant pH. The pH effect on oxygen demand should be investigated further since scale-up of the fermentation process can successfully be achieved in a more economical way if the system requires less oxygen.

Effect of gene expression on cell growth

Apparent specific growth rates of two recombinant strains were summarized in Table 2. It was noted that cells without plasmid grew faster than plasmid-harboring cells as was reported in other works (8-12).

In the case of the strain B (*E. coli* JM109/pTBG 10), the growth rate ratio, $\mu+/\mu-$, under the derepressed condition was rather low (0.626) comparing to that (0.76) of the strain A (*E. coli* MH3000 (pRKcI857)/pAS1(lacZ)). It should be noted that the strain B was cultivated at the constant temperature, 37 °C, while the cultivating temperature for the strain A was elevated from 28 °C to 42 °C under the derepressed condition. Despite this difference, smaller value of $\mu+/\mu-$ of the strain B could be explained by the higher load for the protein synthesis. As shown in the previous section, the strain B produces β-galactosidase about 6 times more than the strain A at its maximum production level.

Effect of gene expression on the plasmid stability

Plasmid stabilities of each recombinant strain were checked for a fermentation period correspon-

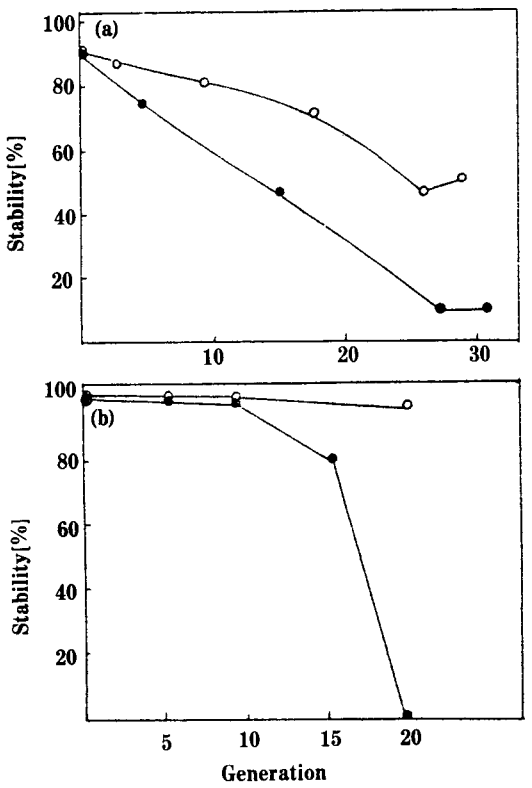

Fig. 5. Stabilities of (a) *E. coli* MH3000 (pRKcI 857)/pAS1 and (b) JM109/pTBG10
○; without induction
●; with induction

ding to 20-30 generations (Fig. 5). Plasmid stabilities of both strains were observed to drop to lower than 10% after about 25 generations, but their decreasing patterns were quite different from each other. Plasmid stability of the strain A under the derepressed condition dropped to 10% in about 25 generations, but decreased at a slower rate under the repressed condition. Compared to this, the plasmid stability of the strain B was observed to be maintained above 95% under the repressed condition even after about 25 generations, while under the derepressed condition about 80% was maintained until 20 generations and then dropped very rapidly.

Conclusion

E. coli JM109/pTBG10 strain which uses *Tac* promoter system had higher productivity and better plasmid stability than *E. coli* MH3000(pRKcI 857)/pAS1(lacZ) strain.

Inductions at initial- or mid-logarithmic growth phases were preferred to late-logarithmic growth phase induction for both strains.

Oxygen demand was found to be very high for the gene expression and could be alleviated to some extent through pH control.

Selection of an appropriate host-vector system with better plasmid stability and strong regulation system was considered to be essential for a successful development of the large-scale fermentation process of recombinant microorganisms.

From this point of view, *E. coli* JM109/pTBG 10 strain was considered to be applicable to the production of hepatitis B-β-galactosidase fusion protein with several improvements at the fermentation level in progress of this work.

요 약

β-Galactosidase 유전자 조작된 두 대장균 숙주-벡터 시스템의 단백질 생산과 배양특성을 비교하였다.

Tac 프로모터를 갖는 *Escherichia coli* JM 109/pTBG 10 균주가 *pL* 프로모터를 갖는 *E. coli* MH 3000(pRKcI 857)/pASI(lacz) 균주보다도 단백질 생산성에 있어서 훨씬 우수하였으며 초기 및 중간 대수 증식기에서의 발현 유도가 단백질 생산에 있어서 유리함을 관찰하였다. 또한 유전자 발현시기에 있어서는 산소요구량이 매우 높았으나 pH를 일정하게 조절함에 의해 산소요구량을 어느 정도 낮출 수 있었고 생산성도 향상됨을 관찰할 수 있었다.

고생산성 균주(*E. coli* JM 109/pTBG 10)의 경우 플라스미드 함유 균체의 플라스미드 상실 균체에 대한 비증식속도의 비 μ^+/μ^- 가 저생산성 균주보다도 낮았으며 이것은 유전자발현에 따른 단백질 합성부담이 매우 큰 때문으로 판단되었다.

또 플라스미드 안정성을 30세대까지 추적하였는바 유전자 발현 조건하에서 두 균주 모두 25세대 후 10% 정도의 안정성을 보였으나 *E. coli* JM 109/pTBG 10 균주의 안정성이 상대적으로 우수하다고 판단되었다.

References

1. Swamy, K.H.S. and Goldberg, A.L.: *Nature* **292,** 652 (1981).
2. O'Farrel, P.H., Polisky, B. and Gelford, D.H.: *J. Bact.* **134,** 645 (1978).
3. Jeffrey H. Miller: Assay of -galactosidase. *In Experiments of Molecular Genetics,* 352 CSH (1972).
4. Johan Botterman and M. Zabeau: *Gene* **37,** 229 (1985).
5. M. Zabeau and K. Stanley: *EMBO J.* **1,** 1217 (1982).
6. E. Remaut, P. Stanssens, and W. Fiers: *Gene* **15,** 81 (1981).
7. J.H. Botterman, D.R. De Buyser, J.A. Spriet, M. Zabeau and G.C. Vansteenkiste: *Biotechnol. Bioeng.* **27,** 1320 (1985).
8. Collins, J. and R.H. Pritchard: *J. Mol. Biol.* **78,** 143 (1973).
9. Engberg, B. and K. Nordstrom: *J. Bacteriol.* **123,** 179 (1975).
10. Inselburg, J.: *J. Bacteriol.* **133,** 433 (1978).
11. Nakazawa, T.: *J. Bacteriol.* **133,** 527 (1978).
12. Godwin, D. and J.H. Slater.: *J. Gen. Microbiol.* **111,** 201 (1979).

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